Acta Biochimica Polonica

Vol. 51 No. 4/2004

861–873

QUARTERLY

Review

Modes of inhibition of cysteine proteases✪

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Received: 22 June, 2004; revised: 23 June, 2004; accepted: 17 October, 2004

Key words: cysteine proteases, proteolysis, inhibitors, staphostatins, cystatins, serpins

Cysteine proteases are involved in many physiological processes and their hyperactivity may lead to severe diseases. Nature has developed various strategies to protect cells and whole organisms against undesired proteolysis. One of them is the control of proteolytic activity by inhibition. This paper presents the mechanisms underlying the action of proteinaceous inhibitors of cysteine proteinases and covers propeptides binding backwards relative to the substrate or distorting the protease catalytic centre similarly to serpins, the p35 protein binding covalently to the enzyme, and cystatins that are exosite binding inhibitors. The paper also discusses tyropins and chagasins that, although unrelated to cystatins, inhibit cysteine proteinases by a similar mechanism, as well as inhibitors of the apoptosis protein family that bind in a direction opposite to that of the substrate, similarly to profragments. Special attention is given to staphostatins, a novel family of inhibitors acting in an unusual manner.

Proteolytic enzymes catalyse the hydrolytic cleavage of peptide bonds. They can be classified as exopeptidases and endopeptidases (proteinases) that are further subdivided into

✪This work was supported in part by the grant 2PO4A 04026 from the State Committee for Scientific Research (KBN, Poland).

⋆Author is awarded a scholarship from The Foundation for Polish Science.

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Abbreviations: BIR, baculovirus IAP repeat; CrmA, cytokine response modifier A; IAP, inhibitors of apoptosis protein; SCCA1, squamous cell carcinoma antigen 1; SpeB, streptococcal pyrogenic exotoxin B.

serine-, aspartic-, metallo- and cysteine proteinases (Barrett *et al.*, 1998). The latter have been found in viruses and prokaryotes as well as in higher organisms such as plants and mammals, including humans. Mammalian cysteine proteinases fall into two classes: caspases (Chapman *et al.*, 1997; Barrett *et al.*, 1998) and the papain superfamily comprising the papain family, calpains and bleomycin hydrolases (Otto & Schirmeister, 1997; Barrett *et al.*, 1998; McGrath *et al.*, 1999).

Cysteine proteinases participate in varied biological processes. The cathepsins alone are involved in protein breakdown in lysosomes, antigen presentation, proteolytic processing of proenzymes and prohormones, fertilization, cell proliferation, differentiation and apoptosis (Chapman *et al.*, 1997; Grzelakowska-Sztabert, 1998; Berdowska & Siewiński, 2000). Imbalanced activity of endogenous cysteine proteinases may lead to numerous pathologies such as rheumatoid arthritis, multiple sclerosis, neurological disorders, tumours and osteoporosis (Berdowska & Siewiński, 2000). The cysteine proteinases produced by pathogenic bacteria are considered important virulence factors and their role in the development of many diseases, e.g. in paradontosis, is postulated (Takahashi *et al.*, 1994; 1999; Pavloff *et al.*, 1995; Potempa *et al.*, 1995; Lowy *et al.*, 1998; Takeuchi *et al.*, 1999; Rice *et al.*, 2001; Dubin, 2002; 2003).

Thus, precise control of proteolytic processes is essential for appropriate functioning of cells and whole organisms. This is achieved at many levels, from regulation of protease expression, secretion and maturation, through specific degradation of mature enzymes, to blockage of their activity by inhibition. Due to possible applications of selective proteinase inhibitors in therapy, the mechanisms underlying inhibition are being thoroughly investigated. This paper reviews the inhibitory mechanisms employed to control the activity of one particular class of proteolytic enzymes, the cysteine proteinases.

STRUCTURE AND CATALYTIC MECHANISM OF CYSTEINE PROTEASES

Before characterizing modes of their inhibition, let us explain the catalytic mechanism of cysteine proteases themselves. The proteases of this group are most commonly exemplified by papain, a well described plant enzyme isolated from the latex of *Carica papaya* fruit. The papain molecule consists of two subdomains forming the active site cleft of the enzyme (Mitchell *et al.*, 1970; Drenth *et al.*, 1976; Garavito *et al.*, 1977; Kamphuis *et al.*, 1984; Varughese *et al.*, 1989).

The proteolytic activity of all cysteine proteases arises from the presence of the catalytic Cys and His residues in the enzyme active centre. In the case of papain-like cysteine proteinases, the catalytic centre is complemented with Asn that ensures an orientation of the His imidazole ring optimal for successive stages of hydrolysis.

The crucial step of the catalytic process involves formation of a reactive thiolate/imidazolium ion pair ($Cys\text{-}S^{-}/His\text{-}Im^{+}$), which results from proton transfer between Cys-25 and His-159 (papain numbering).

In principle, the thiolate anion attacks the carbonyl carbon of the scissile peptide bond, and the double bond between the carbon and the oxygen converts into a single one (Fig. 1A). The oxygen assumes a negative net charge allowing formation of the first tetrahedral transition state. The oxyanion is stabilized by hydrogen bonding to the NH groups of Gln-19 side chain and Cys-25 backbone, which is likely to result in the formation of an oxyanion hole (Fig. 1B) (Menard *et al.*, 1991; Menard *et al.*, 1995; Harrison *et al.*, 1997; Otto & Schirmeister, 1997). Subsequent rotation of the His residue enables proton transfer from the imidazolium cation to the nitrogen of the peptide bond being hydrolyzed, and cleavage occurs. The newly formed substrate amine is hydrogen bonded to His-159,

whereas the substrate carboxylic part is linked to Cys-25 *via* a thioester bond, forming acyl enzyme (Fig. 1C). The next reaction step involves dissociation of the aminic part of the substrate and its replacement with a water molecule. The imidazole nitrogen contributes to polarization of the water molecule that in turn attacks the carbonyl carbon of acyl enzyme (Fig. 1D). This is followed by formation of the second tetrahedral intermediate (Fig. 1E). In the final step, thioester deacylation leads to reconstruction of the carboxyl group in the hydrolyzed substrate, which is concerted with the release of an active enzyme (Fig. 1F) (Menard *et al.*, 1991; Otto & Schirmeister, 1997).

PROPEPTIDES – THE BACKWARD BINDING

A considerable number of cysteine proteinases are synthesized as inactive precursors. Their activation requires proteolytic cleavage of the N-terminal proregion that also functions as inhibitor of the mature enzyme (Groves *et al.*, 1998). Crystallographic studies of procathepsins B, L and K shed light on the propeptide inhibitory mechanism (Fig. 3A) (Turk *et al.*, 1996; Fujishima *et al.*, 1997; Podobnik *et al.*, 1997; LaLonde *et al.*, 1999; Wiederanders, 2003; Wiederanders *et al.*, 2003).

The majority of cysteine protease profragments share similar fold and consist of two parts. The profragment N-terminal portion is built up of two α-helices and an extended β-strand, and interacts with a surface-localised "proregion binding loop" of the mature protease. The C-terminal segment binds between the two domains of the enzyme, being anchored at the S' sites (Schechter and Berger nomenclature, see Fig. 2) by a short α-helix. Its backbone covers the substrate binding site, which hinders access to the enzyme's catalytic centre. However, the binding modes of the substrate and the propeptide are opposite in direction. Al-

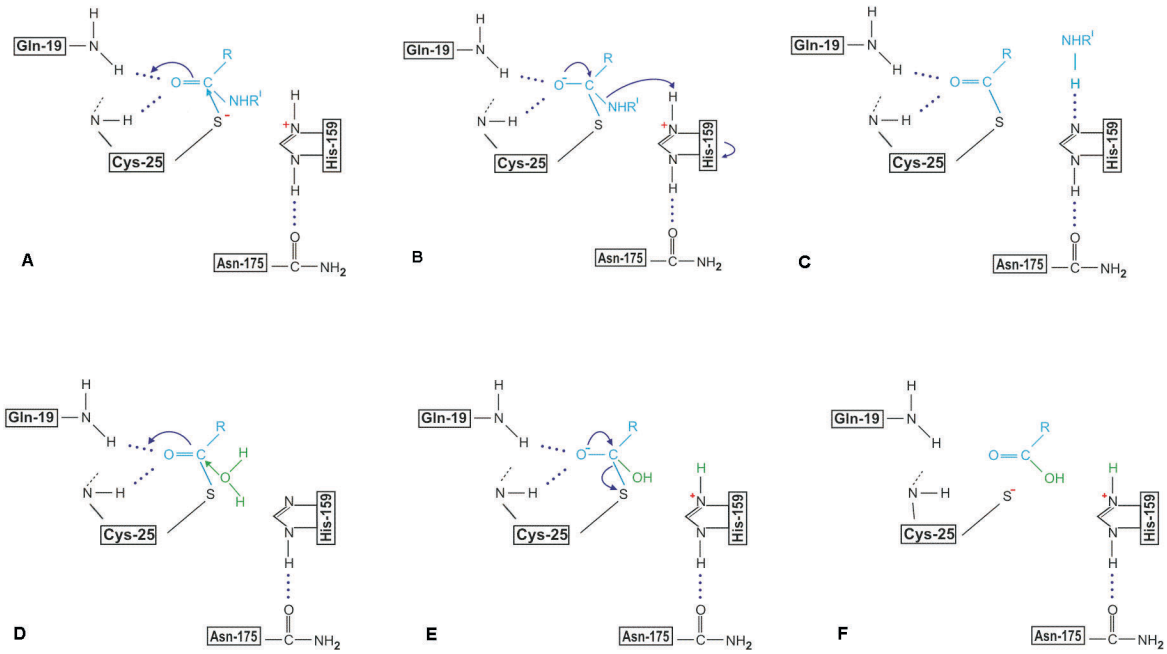

Figure 1. Catalytic mechanism of cysteine proteinases as exemplified by papain (description in the text).

though the propeptide sidechains utilise the same binding sites as the substrates, the reverse orientation of the polypeptide chain results in such a position of the peptide bond that makes it resistant to cleavage (Fox *et al.*, 1992; Carmona *et al.*, 1996; Coulombe *et al.*, 1996; Cygler & Mort, 1997; Podobnik *et al.*, 1997; Wiederanders *et al.*, 2003).

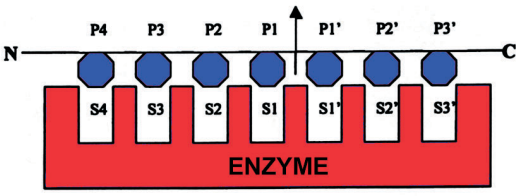

Figure 2.: Schechter and Berger nomenclature.

S4–S3' – substrate binding sites within the active site of the enzyme, P4–P3' – substrate residues that bind to S4–S3' sites, arrow indicates the scissile bond (Schechter & Berger, 1967).

pSpeB – PROFRAGMENT THAT DISTORTS THE ENZYME CATALYTIC CENTRE

An unusual mechanism of proregion inhibition has been described for streptococcal pyrogenic exotoxin B (SpeB), a papain-like protease isolated from *Streptococcus pyogenes* (Fig. 3B). The structure of the SpeB profragment portion (pSpeB) is unique, comprising a four-stranded antiparallel β-sheet flanked by two α-helices. Despite the fact that pSpeB binds the enzyme surface in a position that corresponds to the localisation of the 'proregion binding loop' in procathepsins, its mode of interaction with the protease active site cleft is substantially different (Kagawa *et al.*, 2000). The crystal structure of SpeB zymogen revealed that the predominant interactions with the active site of the mature enzyme are mediated by the first turn of one of the propeptide α-helices. The most crucial in SpeB inhibition is the Asn-89 residue that penetrates the substrate binding groove of the mature enzyme in a position analogous to the S1' site of other papain-like proteases. This results in the catalytic His-195 being pushed out from the active centre, preventing any interactions with the catalytic Cys-47 residue. Consequently, the protease reactive site is distorted and SpeB has no catalytic potency until maturation (Kagawa *et al.*, 2000; Chen *et al.*, 2003).

SERPINS – COVALENT INTERACTION AND CATALYTIC CENTRE DISTORTION

Enzyme inactivation through active site distortion is adopted by other inhibitors as well. Recently, it has been shown that some serpins (serine protease inhibitors) are capable of blocking the activity of both serine and cysteine proteinases. Such inhibitors are usually exemplified by squamous cell carcinoma antigen 1 (SCCA1) targeting the papain-like cathepsins S, K and L (Schick *et al.*, 1998a; 1998b), and the cowpox virus-encoded cytokine response modifier A (CrmA) that selectively inhibits caspases 1 and 8 as well as the serine protease granzyme B (Zhou & Salvesen, 2000; Stennicke *et al.*, 2002).

The crystal structures of complexes between CrmA or SCCA1 and proteases have not been determined so far. The characteristic structural feature of all serpin superfamily members is the presence of a surface exposed reactive site loop that functions as a target for proteases. Since the loop has been found crucial for the inhibitory activity of both CrmA and SCCA1 (Schick *et al.*, 1998a), it is assumed that serpins inhibit both serine and cysteine proteinases in a similar manner.

An insight into the inhibitory mechanism of serpins was provided by the crystal structure of a complex between trypsin, a serine protease, and antitrypsin that belongs to serpins (Huntington *et al.*, 2000). In general, upon interacting with trypsin, the reactive site loop of antitrypsin is cleaved, whereas the catalytic Ser of the enzyme and the carbonyl carbon of the inhibitor P1 residue form an ester

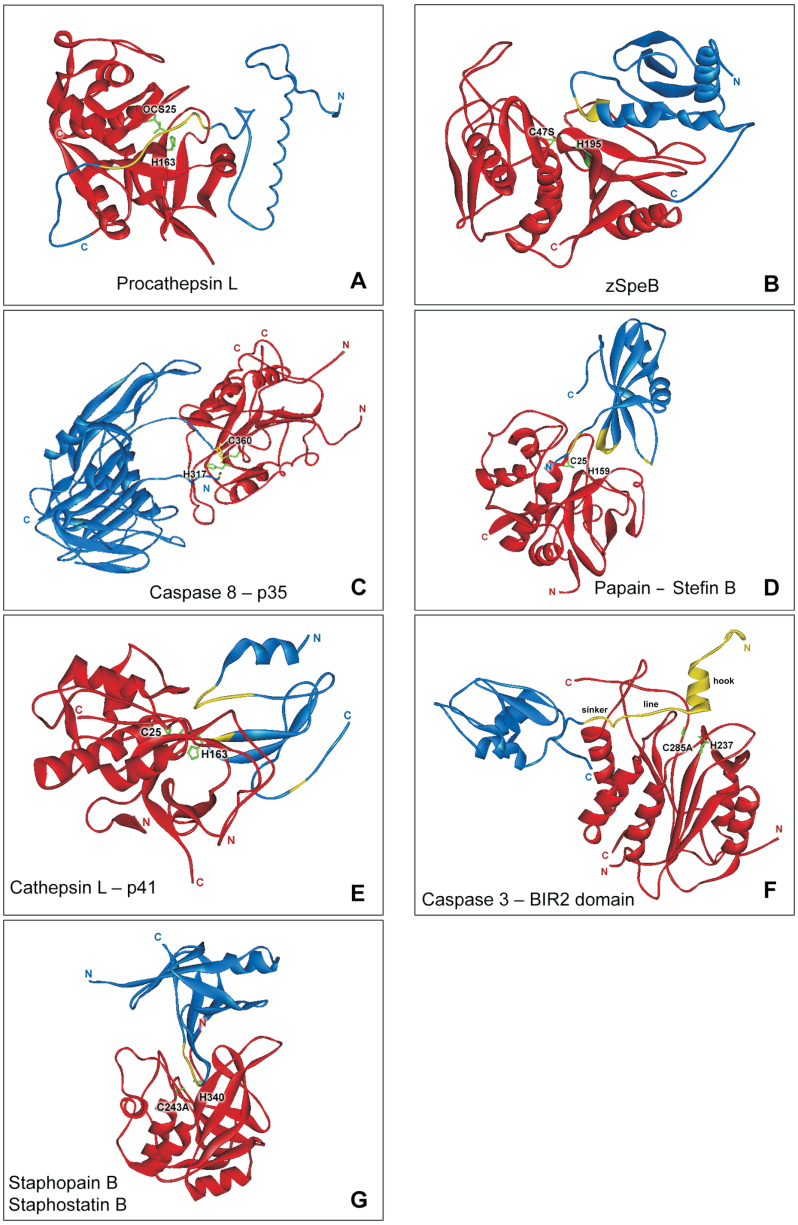

Figure 3. Ribbon drawing of inhibitory complexes formed by A, procathepsin L (PDB code: 1cs8); B, SpeB zymogen (1dki); C, p35 and caspase 8 (1i4e); D, stefin B and papain (1stf); E, p41 and cathepsin L (1icf); F, XIAP and caspase 3 (1i3o); G, staphostatin B and staphopain B (1pxv).

Inhibitors are shown in blue, enzymes in red. The location of the catalytic Cys and His residues is shown in green, regions of inhibitor that are most important for interaction with enzyme are labelled yellow. Figure made with WebLabProViewer.

bond. The newly formed N-terminal part of the loop, covalently linked to the protease, relocates and becomes incorporated into the body of the antitrypsin molecule, which alters the position of the enzyme relative to the inhibitor. This leads to partial denaturation of the protease molecule and disruption of its catalytic centre (Huntington *et al.*, 2000). Due to the enzyme's inability to conduct further catalysis, the acyl intermediate, identical to that observed during substrate hydrolysis, is stabilized and the protease becomes inactive (Stennicke *et al.*, 2002).

Several lines of evidence, e.g. the crystal structure of free CrmA and some biochemical data, suggest that the interaction with

caspase results in the cleavage of the peptide bond within the substrate-like reactive site loop of CrmA. Upon cleavage, the inhibitor undergoes a conformational change into a more stable form rendering the whole process practically irreversible (Stennicke *et al.*, 2002). In the case of caspase inhibition by CrmA, it is supposed that the enzyme catalytic Cys residue links to the inhibitor *via* a thioester bond. The CrmA reactive site loop is shorter than in most serpins, which provides a basis for the assumption that the caspase catalytic Cys, while covalently bound to CrmA, is in a position corresponding to that of the catalytic Ser in the complexes of other serpins with their target serine proteases. Likewise, this might allow deformation of the caspase structure (Simonovic *et al.*, 2000; Ye & Goldsmith, 2001), although in such a case partial denaturation would presumably lead to dissociation of the protease into two subunits (Stennicke *et al.*, 2002).

However, the cross-class serpins seem to differ from other members of the serpin superfamily by their inability to form stable covalent complexes with target cysteine proteinases (Simonovic *et al.*, 2000; Masumoto *et al.*, 2003). Recently, two ways of papain inhibition by SCCA1 have been suggested that comprise the suicide substrate-like mechanism without formation of a covalent complex, and irreversible impairment of papain catalytic activity by disruption of the enzyme's structure (Masumoto *et al.*, 2003).

Nevertheless, the proposed scheme of interaction between serpins and cysteine proteases will be ultimately confirmed upon determination of the structure of at least one serpin–protease complex.

p35 — COVALENT INHIBITION AND STERIC HINDRANCE

The p35 protein is another example of cysteine protease covalent inhibitors. It is produced by baculoviruses in the early phase of infection in order to suppress the host immune response. The members of the p35 protein family have an ability to inhibit almost all known caspases and usually do not exhibit any activity towards the enzymes of other protease families (Stennicke *et al.*, 2002). The structure of free p35 protein consists of four α-helices, a central β-sheet and a reactive site loop exposed over the surface (Fig. 3C) (Ye & Goldsmith, 2001). Based on the structure of human caspase 8 complexed with baculovirus p35 protein, it was discovered that the enzyme inactivation results from the formation of a covalent thioester bond between the caspase 8 catalytic Cys-360 residue and the P1 Asp-87 residue localized within the substrate-like reactive site loop of the inhibitor. Following cleavage of the reactive site loop, the p35 protein undergoes a number of conformational changes that result in the insertion of its N-terminal portion into the enzyme active site cleft. Consequently, the caspase catalytic His-317 residue is inaccessible for solvent molecules, which prevents hydrolysis of the thioester bond. Presumably, at the same time a hydrogen bond is formed between the His-317 catalytic residue and the Cys-2 thiol group of the p35 N-terminus, which leads to an unfavourable rotation of the His-317 imidazole ring and arrests catalysis (Xu *et al.*, 2001).

The p35 protein is distinct from serpins in the mechanism of enzyme inactivation (Xu *et al.*, 2001; Ye & Goldsmith, 2001). The serpins distort the enzyme catalytic centre and rearrange the protease molecule, whereas in the caspase–p35 protein complex the proteolysis is blocked directly by the inhibitor, due to conformational changes within its molecule (Xu *et al.*, 2001).

CYSTATINS — EXOSITE BINDING INHIBITORS

Cystatins constitute the largest and best described group of natural cysteine proteinase

inhibitors (Bode *et al.*, 1990). They are directed against the papain superfamily members found in viruses, bacteria, plants and animals. On the basis of sequence homology, the cystatin superfamily is divided into three groups: stefins (family I), cystatins (family II) and kininogens (family III) (Otto & Schirmeister, 1997; Barrett *et al.*, 1998; Grzonka *et al.*, 2001).

The mechanism of cystatin action was elucidated by numerous kinetic and crystallographic studies (Fig. 3D) (Abrahamson *et al.*, 1987; Abrahamson, 1988; Bode *et al.*, 1988; Hall *et al.*, 1995; Masson *et al.*, 1998; Stubbs *et al.*, 1990). Cystatins are exosite binding inhibitors and they bind adjacent to the protease active site, obstructing the access of substrate, but do not interact with the enzyme catalytic centre directly (Bode & Huber, 2000).

Determination of chicken egg white cystatin structure revealed that the cystatin inhibitory domains are composed of five antiparallel β-strands wrapped around a central α-helix. The molecule scaffold takes the shape of a wedge that fits into the substrate binding groove of papain. The wedge's edge is formed by the partially flexible N-terminus containing the characteristic Gly-8 and Ala-10 residues and two hairpin loops carrying highly conservative motifs QVVAG and PW (Bjork & Ylinenjarvi 1989; Hall *et al.*, 1993; 1995; Grzonka *et al.*, 2001).

The three-dimensional structure of the complex formed between human stefin B and papain conveyed the full understanding of cystatin inhibitory mechanism. The two hairpin loops, typical for cystatins, interact with the enzyme surface at the S1' through S4' binding sites, whereas the N-terminal portion of the cystatin molecule runs through the S3–S1 subsites. Although the polypeptide chain binds in a substrate-like manner, at the P1 position it points away from the enzyme active site and so avoids cleavage. The cystatin molecule remains intact, but still prevents interactions with substrates, being non-productively bound to the enzyme (Stubbs *et al.*, 1990).

THYROPINS AND CHAGASINS – 'CYSTATIN-LIKE' INHIBITION

The very efficient mechanism of inhibition observed in cystatins was also described for inhibitors that belong to protein families not only unrelated to cystatins, but also of an entirely different fold.

The first group of such proteins is formed by thyropins, the protease inhibitors whose structures contain an arrangement designated as thyroglobulin type-1 domain (Lenarcic *et al.*, 1999). Crystallographic analyses of major histocompatibility complex class II-associated p41 invariant chain bound to cathepsin L provided the molecular background for the mechanism of interaction between thyropins and their target proteases (Fig. 3E) (Guncar *et al.*, 1999). The p41 fragment consists of two subdomains: the first one composed of an α-helix and a β-strand, the second subdomain being a three-stranded antiparallel β-sheet. Similarly to cystatins, the molecule is wedge-shaped and interacts with the enzyme through three hairpin loops (Guncar *et al.*, 1999). However, the different overall structure results in additional contacts with the surface of the protease, determining high specificity of thyropins and distinguishing them from the relatively non-selective cystatins (Guncar *et al.*, 1999; Bode & Huber, 2000).

The chagasin family members presumably share a comparable mode of inhibition. Chagasins were first identified in *Trypanosoma cruzi* and they are inhibitory towards papain-like proteinases of bacterial, protozoan and mammalian origin (Monteiro *et al.*, 2001; Rigden *et al.*, 2002; Sandersen *et al.*, 2003). Although the three-dimensional structure of any chagasin is so far unknown, computer based predictions revealed that it should have an immunoglobulin-like fold and

contain a sequence motif corresponding to two adjacent β-strands connected *via* a short turn. This suggests a wedge-like structure resembling that inserted into the papain and cathepsin L active sites by stefin B and p41 (Monteiro *et al.*, 2001; Rigden *et al.*, 2002; Sandersen *et al.*, 2003).

IAP – PROTEINACEOUS INHIBITORS THAT BIND BACKWARDS

The next noteworthy group of cysteine protease inhibitors comprises inhibitors of the apoptosis protein family (IAP), endogenous proteins that function through direct inhibition of caspases. Their most distinguishable characteristic is the subunit structure with one or more BIR (baculovirus IAP repeat) domains.

On the basis of the three-dimensional structures of complexes between human XIAP BIR2 domain and caspases 3 or 7 it has been established that the BIR domain does not contribute directly to the protease inhibition, whereas all prominent contacts are mediated through the flexible portion of the XIAP polypeptide chain preceding the BIR domain. This portion consists of the regions designated 'hook', 'line' and 'sinker', and proceeds along the enzyme active site cleft in a direction opposite to that of substrates (Fig. 3F).

The mechanism of caspase 3 and 7 inhibition is based on sterically hindering the substrate access to the enzyme catalytic centre, mostly *via* non-covalent interactions of the helical 'hook' with the outer edge of the substrate canyon, while the BIR domain and the 'sinker' region are responsible for stabilization of the inhibitory complex (Chai *et al.*, 2001; Riedl *et al.*, 2001; Stennicke *et al.*, 2002). However, the most remarkable and unique feature of the mode of caspase 3 and 7 inhibition by XIAP is the backward binding accompanied by the lack of interactions within the S1 substrate binding site.

STAPHOSTATINS – A NEW FAMILY OF INHIBITORS

Recently, a new mechanistic class of cysteine proteinases inhibitors has been identified. They were named staphostatins due to their high specificity towards staphopains, bacterial papain-like cysteine proteases (Rice *et al.*, 2001; Massimi *et al.*, 2002; Rzychon *et al.*, 2003a). The staphostatins were found to be β-barrels formed by a three-stranded mixed β-sheet and a five-stranded anti-parallel β-sheet (Dubin *et al.*, 2003; Rzychon *et al.*, 2003b).

The crystal structure of staphostatin B in complex with staphopain B (Fig. 3G) revealed that their most crucial interactions occur in the region defined as the inhibitor binding loop. The loop spans the protease active site cleft in a direction analogous to that of substrates (Filipek *et al.*, 2003). An essential role in the inhibitory mechanism is played by the Gly-98 residue localized in the P1 position and conserved in all staphostatins sequences. It adopts an unusual, strained backbone conformation that would be unavailable to any other residue for steric reasons. This conformation results in the O-C-S angle (made by the carbonyl oxygen and carbonyl carbon of the inhibitor P1–P1' peptide bond, and sulphur of the enzyme's catalytic Cys) approaching 180°, far too high a value for effective nucleophilic attack. Moreover, the position of the carbonyl oxygen of the P1–P1' peptide bond, relative to the oxyanion hole-forming side chain NH of Gln-19 and backbone NH of Cys-25, is distinct from that anticipated for the substrate, which prevents stabilization of the tetrahedral intermediate. This blocks proteolysis in its initial stage and renders the enzyme inactive (Dubin *et al.*, 2003; Filipek *et al.*, 2003).

SUMMARY

All proteinaceous inhibitors of proteinases, including cysteine proteinase inhibitors, act

through steric blockage of the substrate access to the enzyme catalytic centre. Nature has developed a number of strategies to achieve this goal (Fig. 4).

Most profragments follow the tactics of spanning the enzyme active site in the direction opposite to that of substrates. Upon binding, the resulting peptide bond position prevents hydrolysis of the proregion, as the propeptide chain interposes a hindrance between the substrate and the catalytic centre of the protease. A seemingly analogous inhibitory mechanism is employed by proteins of the IAP family that also occupy the enzyme's active site in a non-productive orientation.

An unusual mode of profragment inhibition has been reported for pSpeB which distorts the protease catalytic centre. Similarly, members of the serpin superfamily inactivate target proteases by deformation of their active sites. Moreover, they bind covalently and enforce rearrangement of the entire enzyme molecule.

The inhibitory mechanism utilized by serpins as well as the p35 protein is commonly compared to a mousetrap. Inhibitor (the trap) is initially treated as substrate but in the course of reaction the transition state gets stabilized and the enzyme (the mouse) is trapped (Stennicke *et al.*, 2002). However, unlike serpins, the p35 protein does not reorganize the protease structure but directly blocks further proteolysis.

Three groups of structurally and evolutionarily distinct protease inhibitors — cystatins, thyropins and chagasins — adopt a common strategy. They all bind adjacent to the active centre and block the access of the substrate indirectly, without engaging the enzyme's catalytic residues.

Finally, yet another mechanism of action is employed by staphostatins that bind directly to the active site, still avoiding cleavage due to the unusual conformation of the P1 residue.

The proteinaceous inhibitors of proteolytic enzymes function as regulators of endogenous proteases and contribute to protection against the detrimental consequences of excessive exogenous protease activity. Detailed understanding of the inhibitory mechanisms they employ may provide prospects for treatment of the diverse disorders that result from defective control of proteolytic processes.

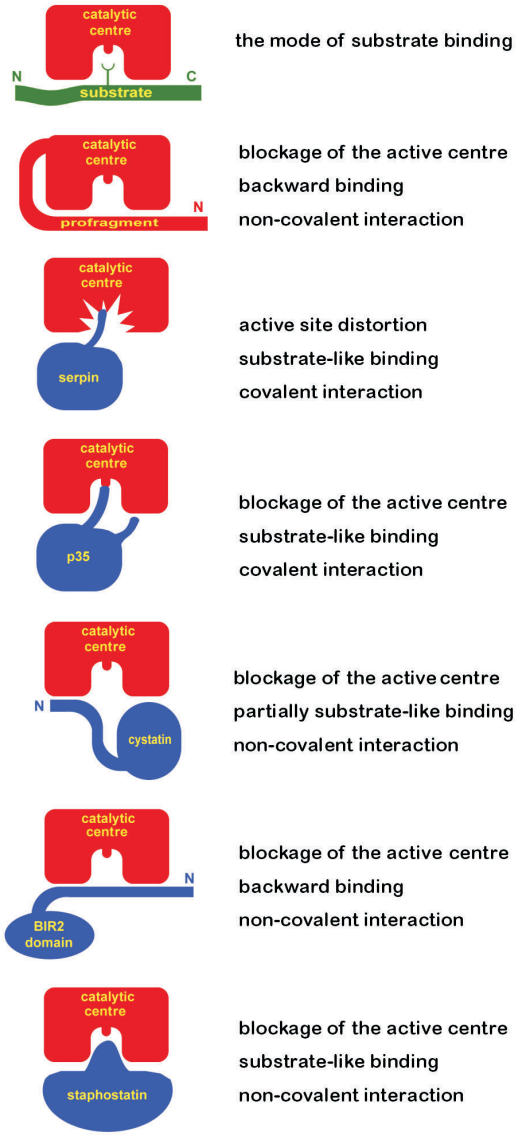

Figure 4. Schematic representation of inhibitory mechanisms directed against cysteine proteinases.

Inhibitors are shown in blue, enzymes in red, substrate in green.

The authors gratefully acknowledge Prof. Adam Dubin and Tomasz Dylag for critical reading of the manuscript.

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